Research Article

Induction of Therapeutically Relevant Cytotoxic T Lymphocytes in Humans by Percutaneous Peptide Immunization

Hiroaki Yagi,[1] Hideo Hashizume,[1] Takahiro Horibe,[1] Yasushi Yoshinari,[1] Maki Hata,[1] Akihiro Ohshima,[1] Taisuke Ito,[1] Masahiro Takigawa,[1] Akihiko Shibaki,[2] Hiroshi Shimizu,[2] and Naohiro Seo[1]

[1]Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan and [2]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Abstract

Percutaneous peptide immunization (PPI) is a simple and noninvasive immunization approach to induce potent CTL responses by peptide delivery via skin with the stratum corneum removed. After such a barrier disruption in human skin, epidermal Langerhans cells, although functionally matured through the up-regulation of HLA expression and costimulatory molecules, were found to emigrate with a reduced number of dendrites. $CD8^+$ populations binding to MHC-peptide tetramers/pentamers and producing IFN-γ appeared in the blood after PPI with HLA class I–restricted antigenic peptides. PPI with melanoma-associated peptides reduced the lesion size and suppressed further development of tumors in four of seven patients with advanced melanoma. These beneficial effects were accompanied by the generation of circulating CTLs with *in vitro* cytolytic activity and extensive infiltration of tetramer/pentamer-binding cells into regressing lesions. PPI elicited neither local nor systemic toxicity or autoimmunity, except for vitiligo, in patients with melanoma. Therefore, PPI represents a novel therapeutic intervention for cancer in the clinical setting. (Cancer Res 2006; 66(20): 10136-44)

Introduction

Dendritic cell (DC)-based immunotherapy for cancer and infection is feasible, safe, and effective in some cancer patients when appropriately matured and activated DCs are administered (1–11). However, because of the lack of standardization in methods for *in vitro* generation of DCs and protocols to administer these vectors, comparison of clinical efficacy among DC immunotherapies, and thus, designing large clinical trials seems to be difficult (12). More critically, the intricate processes involved are costly and time-consuming, constituting an obstacle to clinical application of DC-based strategies. Targeting DCs should be ideally done *in vivo* (12, 13).

Epidermal Langerhans cells (LC), immature DCs residing in the outermost layer of the skin, become potent antigen-presenting cells (APC) after appropriate stimulation (14, 15). Because of their unique anatomic localization and immune functions, LCs are very attractive vectors for vaccine delivery (14, 16–19). Percutaneous peptide immunization (PPI) represents a novel immunotherapy using LCs as vectors for delivering all classes of peptide to the immune system. Removal of the stratum corneum (SC), the most superficial layer of the epidermis, by physical means, is essential in this simple and noninvasive method (20). The barrier removal process not only enhances the permeability of antigenic peptides applied to the skin but also matures LCs for antigen presentation (21). Following migration from barrier-disrupted skin to lymphoid organs, LCs bearing peptides induce potent CTL responses. Experiments in murine tumor models have shown that this simple and safe procedure is highly effective for prophylaxis and therapy of infection and tumors (19, 20, 22). The present study was designed to validate PPI as a clinical intervention for the management of malignancies in humans.

Materials and Methods

Human Subjects

A total of eight healthy males, with ages ranging from 24 to 56 years, and eight patients with stage III/IV melanoma (P1-P8; Table 1) were enrolled in the present study. Three healthy males, designated N1 (age, 35 years), N2 (40 years), and N3 (30 years), all having HLA-A*0201, underwent PPI with HIV gag peptides. Another six healthy subjects, with ages ranging from 24 to 56 years, participated in clinical and immunohistochemical studies following the removal of SC but did not receive PPI. Patients with melanoma received PPI with melanoma-associated antigenic peptides. Eligible criteria for PPI in the patients were: biopsy-proven American Joint Committee on Cancer stage III/IV melanoma; age, ≥20 years; Eastern Cooperative Oncology Group performance status, ≤1 on entry of PPI; HLA-A*0201 and/or HLA-A*2402 phenotype; normal blood CD4 and CD8 T cell numbers by flow cytometry; and normal quantitative immunoglobulin levels. Exclusion criteria were: prior chemotherapy or application of biologicals ≤4 weeks before trial entry, untreated lesions in the central nervous system, bulky hepatic metastatic lesions, pregnancy, and concurrent corticosteroid/immunosuppressive therapy. Patients with generalized inflammatory skin disease, autoimmune disease, or active infections, including viral hepatitis, were also excluded. P1 to P4, P7, and P8 developed metastatic lesions despite initial treatment involving wide local excision and therapeutic lymph node dissection followed by postoperative combination adjuvant therapy (23). P6 developed primary esophageal melanoma. Due to resection of all the skin lesions and metastatic lymph nodes, P1 was free from measurable lesions on entry and served for evaluation of CTL induction only. In contrast, due to the presence of measurable lesions on entry, both CTL induction and early clinical outcome were assessed in P2 to P8.

In all subjects, routine blood examinations including a hemogram, and assessment of liver and renal function and autoantibodies showed no abnormalities on entry to PPI treatment. Serologic tests for HIV were negative, whereas serum hemagglutination inhibition titers for influenza A virus showed positive results. This study was approved by the institutional

Note: H. Yagi and H. Hashizume contributed equally to this work.

Requests for reprints: Hiroaki Yagi, Department of Dermatology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan. Phone: 81-53-435-2303; Fax: 81-53-435-2368; E-mail: hiroyagi@hama-med.ac.jp.

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review board. Informed consent was obtained from all participants according to the Declaration of Helsinki.

Synthetic Peptides and Reagents

Three custom-synthesized peptides including HIV gag (SLYNTVATL), influenza A matrix protein (MP; GILGFVFTL; American Peptide Company, Inc., Sunnyvale, CA), and modified Melan-A immunodominant cells (ELAGIGILTV; Peptide Institute, Inc., Osaka, Japan; ref. 24), were used as HLA-A*0201-restricted epitopes and four custom-synthesized peptides including MAGE-2 (EYLQLVFGI), MAGE-3 (IMPKAGLLI), gp-100 (VWKTWGQYW), and tyrosinase (AFLPWHRLF; Peptide Institute) were used as HLA-A*2402-restricted epitopes. Their purity was >95.0% as confirmed by high-pressure liquid chromatography. Phycoerythrin (PE)-labeled MHC tetramers specific for the HIV gag and Melan-A peptides were purchased from Beckman Coulter (Villepinte, France) and PE-labeled MHC-pentamers for MAGE-2, MAGE-3, and tyrosinase were custom-synthesized by ProImmune Limited (Littlemore, United Kingdom). The monoclonal antibodies (mAb) used in this study were anti-S-100 protein (DAKO, Glostrup, Denmark), anti-DC-LAMP/CD208 (Beckman Coulter), anti-Langerin/CD207 (Vector, Burlingame, CA), PE-labeled or PerCP-labeled anti-HLA-DR, FITC-labeled anti-CD4, PE-labeled or PerCP-labeled anti-CD8 and PerCP-labeled anti-CD45 (BD Biosciences, San Jose, CA), PE-labeled anti-CD1a, FITC-labeled anti-HLA-ABC (pan-HLA class I), anti-CD80, and anti-CD86 (PharMingen, San Diego, CA). RPMI 1640 complete was used for culture medium (20).

Epidermal Barrier Disruption

To remove SC, 5 × 5 cm square plastic plates were painted evenly with ~100 mg/plate of cyanoacrylate (Aron alpha A, Sankyo, Japan), tightly attached to the skin for 3 minutes and removed gently. This procedure was repeated thrice at one spot.

Transepidermal Water Loss Evaluation

Transepidermal water loss (TEWL) was measured using a Tewameter TM210 (Courage + Khazaka Electronic GmbH, Köln, Germany) as previously described (21).

PPI

Solutions of immunization peptides were made immediately before use. HLA-A*0201 subjects received 10 mg of HIV gag peptide in 10 mL of PBS or 16 mg of Melan-A peptide in 8 mL of 5% DMSO in PBS (5% DMSO). A cocktail of 5 mg each of MAGE-2, tyrosinase, and gp-100 peptides in 10 mL of PBS, and 4 mg of MAGE-3 peptide in 2 mL of 5% DMSO, were used for HLA-A*2402 subjects. These concentrations represented the saturation points of the peptides at room temperature. In P3 and P8, who had both HLA-A*0201 and HLA-A*2402, the MAGE-3 peptide solution contained 4 mg of Melan-A peptide. Twenty-four hours after the removal of SC, these peptide solutions were soaked up by gauze pads (each 5 × 5 cm) and applied to four barrier-disrupted areas (total area, 100 cm^2) of HLA-A*0201 subjects and to the six areas (total area, 150 cm^2) of both HLA-A*2402 subjects and individuals with both alleles. The pads were immediately covered with a film dressing and removed 24 hours later. PPI was repeated six times in normal volunteers and seven times in patients with melanoma at monthly intervals by placing the patches at different sites of the arms, thighs, abdomen, and back. Assessment of hemograms, and liver and renal functions was done 1 week following each PPI and at 1- to 3-month intervals thereafter.

Skin Specimens and Epidermal Cell Suspensions

Biopsy specimens were processed for routine histology and immunohistochemistry (25). The epidermal sheets were separated from the dermis in 0.02 mol/L of EDTA-PBS at 4°C for 18 hours and subjected to immunohistochemical staining. Epidermal suspensions were prepared from blister roofs by limited trypsinization (26).

Identification of LCs

Cells expressing S-100 protein in epidermal sections, and those positive for HLA-DR and CD1a in epidermal sheets and suspensions were identified as LCs (14, 27). Immunostained epidermal sheets were observed in a confocal laser scanning microscope (MRC-600; Bio-Rad, Hercules, CA; ref. 27). Epidermal cell suspensions were stained with a PE-labeled anti-CD1a mAb, a PerCP-labeled anti-HLA-DR mAb, and any one of an FITC-labeled anti-HLA-ABC mAb, an FITC-labeled anti-CD80 mAb, an FITC-labeled anti-CD83 mAb, an FITC-labeled anti-CD86 mAb, or FITC-labeled control antibodies and subjected to flow cytometry.

Flow Cytometry

Samples were run on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Software as described (28, 29). Three × 10^4 and 2 × 10^5 events were analyzed for epidermal samples and blood cells, respectively. For analysis of the epidermal samples, only size gates were used for counting the total number of HLA-ABC$^+$ epidermal cells. Gates for LCs were set as described in the legend for Fig. 1.

Measurement of Immune Responses

Peripheral blood mononuclear cells (PBMC) were purified before and 7 days after each PPI by standard Ficoll density centrifugation and subjected to flow cytometric analysis for cell surface staining and intracellular IFN-γ expression. Because binding to tetramers and pentamers and IFN-γ staining are highly reproducible with a variation of <5%, when the number of positive cells was +2 SD above the mean background staining, we defined this as the specific induction of antigen-specific CTLs.

Cell culture. PBMCs (5 × 10^6 cells/well in complete medium in 12-well tissue culture plates) were stimulated with immunization peptide (10 μg/mL), influenza A MP (10 μg/mL), or Con A (1 μg/mL; Sigma-Aldrich, St. Louis, MO) for 5 days and underwent either tetramer or pentamer staining and *in vitro* cytotoxic assay. Alternatively, cells cultured for 48 hours were subjected to intracellular IFN-γ production. Control cultures contained no stimulant. PBMCs from HLA-A*0201 or HLA-A*2402 subjects without PPI were cultured under identical conditions.

MHC tetramer and pentamer staining. Fresh or cultured PBMCs were stained with PE-labeled HLA-A*0201 tetramers or PE-labeled HLA-A*2402 pentamers and a gating kit (Beckman Coulter) according to the manufacturer's directions.

Intracellular IFN-γ analysis. GolgiStop (0.7 μL/mL, PharMingen) was added to cultures 8 hours before harvesting. Cells were reacted with a PerCP-conjugated anti-CD8 mAb, permeabilized in CytoFix/Cytoperm plus Perm/Wash buffers (PharMingen), and stained with an FITC-labeled anti-IFN-γ mAb (PharMingen). Control levels were determined with an appropriate isotype-matched antibody in each experiment.

***In vitro* peptide-dependent cytotoxic assay.** Only cultures containing >5% of tetramer-positive or pentamer-positive cells among CD8$^+$ cells following peptide stimulation were further expanded with rIL-2 (10 units/mL) for 3 days for effector cells. T2-A24 target cells (1 × 10^6 cells; T2 cell line transfected with HLA-A*2402 gene; ref. 30) incubated with immunizing peptide at 1 μg/mL for 1 hour were subjected to both HLA-A2-restricted and HLA-A24-restricted, calcein-AM release CTL assays (Dojindo Lab., Kumamoto, Japan). Briefly, T2-A24 cells were labeled with 5 μmol/L of calcein-AM in serum- and phenol red–free Iscove's modified Dulbecco's medium (IMDM; Invitrogen Co., Carlsbad, CA) at a concentration of 2 × 10^6 cells/mL for 40 minutes. Effector and target cells in 200 μL of IMDM supplemented with 10% FCS were distributed into U-bottomed 96-well microtiter plates at effector/target ratios of 2, 5, and 10. After incubation at 37°C for 3 hours, the concentration of calcein-AM in the medium was measured in a fluorescence analyzer (Synergy HT, Bio-Tek Inst., Inc., Winooski, VT). Maximal lysis was determined by adding lysis buffer to target cells and percentage-specific lysis were calculated as described previously (31).

Therapeutic PPI and Assessment of Early Clinical Outcome

Based on the kinetics of CTL generation in the blood, we instituted a treatment plan of PPI for patients with melanoma consisting of immunization with melanoma-associated peptide, seven times in total, done once a month. All baseline evaluations were done on entry. Tumor responses and side effects were assessed based on physical examination and laboratory investigation after completion of PPI. To assess tumor responses, the size of target lesions selected according to the Response Evaluation Criteria in Solid Tumors guidelines (32) was measured before and after PPI. Imaging-based evaluations were analyzed using NIH Image software for digital images. In P8, the dot densities in ^{99m}technetium

Table 1. Patient characteristics, disease status on entry and after PPI, and immunologic and clinical responses to PPI

Patient ID*	Age/gender	HLA-A		Stage/months after staging/previous therapy	Status/measurable disease on entry/number of target lesions	% CD3+ CD8+ T cells in peripheral blood†	Number of peptide-specific T cells/10^4 CD8+ cells in pre-PPI/post-PPI blood samples	
		02/01	24				Melan-A‡	Tyrosinase‡
P1	53/F	+	—	Stage IV/48/surgery, DAV-feron	CR/surgical removal of all skin and LN lesions/0	20.4	2/36	ND
P2	78/F	—	+	Stage III/7/surgery, DAV-feron	PD¶/surgical removal of all skin and LN lesions/0	19.8	ND	12/53
P3	77/M	+	+	Stage IV/20/surgery, DAV-feron	PD/mediastinal LN mass by CT and ^{67}Ga scintigraphy/1	16.8	2/94	11/61
P4	20/M	—	+	Stage IV/7/surgery, DAV-feron	PD/skin, lung and liver nodules by CT and MRI/10	27.2	ND	19/22
P5	68/F	—	+	Stage IV/5/surgery	PD/s.c. nodules by CE and CT/5	21.7	ND	15/14
P6	52/M	—	+	Stage III/2/none	PD/esophageal mass by CT and endoscopy/1	20.5	ND	6/105
P7	63/M	+	—	Stage IV/6/surgery, DAV-feron	PD¶/s.c. nodule by CE/1	24.2	1/118	ND
P8	77/M	+	+	Stage IV/5/surgery, DAV-feron	PD/multiple bone metastases by ^{99m}Tc bone scintigraphy/6	17.2	4/113	9/75

Abbreviations: CE, clinical examination; CR, free from melanoma for >4 weeks before entry; CT, computed tomography; DAV-feron, adjuvant therapy with i.v. dacarbazine, nimustine hydrochloride, and vincristine plus lesional injection of IFN-β; LN, lymph node; MRI, magnetic resonance imaging; PD, progression of measurable disease and/or new lesions; ND, not done.

*Patient ID, patient identification number.

† Percentages after lymphocyte gating by flow cytometry.

‡ Number of cells positive for MHC tetramers or pentamers/10^4 CD8+ T cells in freshly isolated PBMCs.

§Number of intracellular IFN-γ-positive cells/10^4 CD8+ T cells in PBMCs stimulated with gp-100 peptide (10 μg/mL) for 48 hours and subjected to an intracellular IFN-γ assay.

P2 and P7 developed new metastatic skin nodules at frequencies of one and three per 2 months, respectively, before entry.

¶In P4 and P5, the sums of the longest diameters for 10 and 5 lesions, respectively, were measured as the pre-PPI and post-PPI sums of the longest diameters.

**The dot densities in ^{99m}technetium hydroxymethylene diphosphonate bone scintigraphs of the metastatic lesions (indicated in Fig. 4) pre-PPI/post-PPI were 98.4/60.8 (#1), 128.6/59.8 (#2), 72.3/55.1 (#3), 84.3/71.3 (#4), 135.8/118.5 (#5), and 101.3/100.8 (#6). The serum 5-S-cysteinyldopa level reduced from 27 nmol/L pre-PPI to 4.2 nmol/L post-PPI (normal, <8.0 nmol/L).

hydroxymethylene diphosphonate bone scintigraphs were compared before and after PPI. The mean background density was calculated based on the density of three unaffected areas in a scintigraph. After the mean background densities before and after PPI were equalized, the lesional density was determined by analyzing the densities of square-framed areas representing bone metastasis.

Statistical Analysis

Student's t test was employed for comparisons, with $P < 0.05$ considered significant.

Results

Clinical observations after removal of sc. After removal of SC, faint and transient erythema developed in the treated sites which disappeared within 1 hour. Subjective symptoms such as pain or skin irritation during or after the manipulation were minimal or absent.

Kinetics of LCs in barrier-disrupted skin. Tissue histology revealed that 60% to 80% of the SC was removed in normal subjects ($n = 3$), on comparison of the thickness of SC before and immediately after barrier disruption, using adjacent skin as a control (Fig. 1*A*). TEWL values significantly increased from 8.10 ± 0.66 before manipulation to 16.83 ± 2.20 g/m^2/h after the removal of SC ($n = 3$, $P < 0.05$), indicating that considerable barrier disruption occurred with the method employed. Recovery was already evident at 48 hours and there were minimal or no inflammatory responses after 12, 24, and 48 hours as revealed by tissue histology. S-100+ LCs showed larger cell bodies with fewer dendrites after the removal of SC compared with their

Table 1. Patient characteristics, disease status on entry and after PPI, and immunologic and clinical responses to PPI (Cont'd)

Number of peptide-specific T cells/10^4 $CD8^+$ cells in pre-PPI/post-PPI blood samples			Sums of the longest diameters of pre-PPI/post-PPI target lesions (cm)	Clinical outcome after PPI/side effects	Follow-up
MAGE-2[‡]	MAGE-3[‡]	gp-100[§]			
ND	ND	ND	0/0	No new lesions/generalized, progressive vitiligo	Disease-free for 19 months after PPI
6/68	9/106	6/30	0/0	No new lesions/none	Three new s.c. nodules (<5 mm in size) at 3 months after first round of PPI, which disappeared after the second round of PPI
8/42	14/212	4/23	3.2/0.9	Normal LN size/generalized, progressive vitiligo	Disease-free for 15 months after PPI
6/34	22/189	4/3	22/32[¶]	New metastatic lesions/none during PPI	Early death due to melanoma lesions
4/34	14/206	8/9	17/8[¶]	Decrease in size of target lesions/generalized, progressive vitiligo	New cutaneous and metastatic lesions developed despite continuous immunization for 13 months
8/93	8/204	12/243	4.0/5.5	Enlargement of a lesion/ generalized vitiligo	Death due to melanoma lesions at 4 months after PPI
ND	ND	ND	2/0	No new lesions/none	Two new s.c. nodules (<5 mm in size) at 5 months after first round of PPI, which disappeared after second round of PPI
18/41	16/94	5/75	Reduced intensities of the scintigraphy signals**	No new lesions/none	New lesion at 4 months after PPI despite unchanged densities of previous lesions

counterparts in intact skin. Both epidermis and dermis at 12 and 24 hours harbored S-100^+ cells, whereas most of them were located in the dermis at 48 hours. HLA-DR^+ cells were found to be larger and stained more brightly at 12 and 24 hours in epidermal sheets (Fig. 1*A*, *inset*) as compared with the intact skin case. At 48 hours, Langerin$^+$ cells were distributed in both epidermis and dermis, whereas DC-LAMP$^+$ cells were found mainly in the dermis. Enumeration of CD1a$^+$ cells in HLA-ABC$^+$ epidermal cell suspensions showed that essentially all LCs remained in the epidermis at 12 and 24 hours, and about half of this population migrated into the dermis at 48 hours (Fig. 1*B*). Enumeration of LCs in the epidermal sheets of three individuals showed that the mean number of HLA-DR^+ cells were 83% of the intact skin case at 24 hours and 39% of the cells remained in the epidermis at 48 hours. In accordance with these morphologic observations, the expression of HLA-ABC and HLA-DR (Fig. 1*C*) was up-regulated and the numbers of $CD80^+$, $CD83^+$, and $CD86^+$ (Fig. 1*D*) cells in LC populations were increased at 12 and 24 hours. The cells expressing CD86 remained increased in number even at 48 hours. Fully mature LCs are recognized by their strong surface expression of MHC class I/class II, CD80, CD86 costimulatory molecules, and CD83 maturation markers (33). Therefore, these findings indicated LC subpopulations to be activated *in situ*, then emigrating from the epidermis following disruption of the epidermal barrier by removal of SC.

Induction of antigenic peptide-specific CTLs by PPI. Based on the LC kinetics, we reasoned that optimal CTL priming might be induced by the application of antigenic peptides 24 hours after barrier disruption and subsequent exposure of the skin sites to the peptides for 24 hours. Normal subjects (N1, N2, and N3) received PPI with the HIV gag peptide. Six patients with stage IV melanoma (P1, P3, P4, P5, P7, and P8) and two patients with stage III melanoma (P2 and P6) underwent PPI with melanoma-associated antigenic peptides. Application of peptide at SC-removed sites was well tolerated, without local reactions such as irritation, redness and erosion, or systemic toxicity evidenced by rashes, fatigue, or fever. None of the study participants experienced lymphadenopathy thought to be related to PPI.

The appearance of CTLs was assessed periodically in the peripheral blood with reference to cells positive for tetramers and pentamers and intracellular IFN-γ^+ cells. Representative data of CTL induction in normal subjects and melanoma patients are shown in Figs. 2 and 3. Tetramer/pentamer-positive $CD8^+$ T cells were successfully detected in freshly obtained blood when assayed 7 days after the fourth immunization with HIV gag in cases N1 to N3, and after the fifth to sixth immunization with melanoma-associated peptides in patients P1 to P3 (Fig. 2*A-C*). The frequencies of binding cells were maintained in N1 and N2, whereas frequencies were increased in N3, P1, P2, and P3 following repeated immunization. Such *in vivo* expansion of CTL in normal individuals and patients with melanoma was antigen-specific because tetramer-binding responses were apparently enhanced in cultures stimulated with immunizing peptide but not with nonimmunizing peptide (Fig. 2*D*). The generation of HLA-A*0201-restricted CTLs was also detected with Melan A-specific tetramers in P7 and P8. After completion of therapeutic PPI, P2, P3, P6, P7, and P8 developed $CD8^+$ T cells reactive with pentamers for tyrosinase, MAGE-2, and MAGE-3, whereas only MAGE-2-specific

and MAGE-3-specific binding occurred in P4 and P5 (Table 1). Application of the HIV gag peptide to intact skin for 24 hours done once a month for five times did not induce CTL responses in N1 and N2. These data clearly indicate that repeated immunization induces and maintains antigenic peptide-specific CTLs.

Antigenic peptide-specific production of intracellular IFN-γ. Stimulation with Con A and Melan-A peptide induced significant numbers of intracellular IFN-γ^+CD8^+ T cells in patients undergoing PPI (Fig. 3*A*). PPI induced $CD8^+$ T cells with intracellular IFN-γ after the fifth immunization with the HIV gag in N1 to N3, and after the sixth and fourth immunization with Melan-A in P1 and P3, respectively (Fig. 3*B*). In addition, $CD8^+$ T cells positive for intracellular IFN-γ were expanded after stimulation *in vitro* with influenza A MP in HLA-A*0201-positive participants who were revealed to have functional antibodies for influenza A viruses by serologic studies. These observations not only indicated the presence of functional CTLs in the peripheral blood but also confirmed the feasibility of the present assay for detecting IFN-γ-producing cells. Four of six HLA-A*2402-positive melanoma patients (P2, P3, P6, and P8) developed gp-100-specific T cells positive for intracellular IFN-γ at completion of therapeutic PPI (Table 1). Therefore, we concluded that repeated immunization in PPI induces and maintains immunologically active, peptide-specific CTLs.

***In vitro* cytolytic function of PPI-induced CTLs.** In cytotoxic assays at completion of PPI, PBMCs from P2, P3, and P5 exerted a marked antigen-specific killing activity against HLA-A*2402 peptide-pulsed T2-A24 cells. Figure 3*C* shows the results of representative experiments with tyrosinase in P2. Effector cell populations contained 8.2%, 7.3%, and 6.7% peptide-matched pentamer-positive cells among $CD8^+$ cells in P2, P3, and P5, respectively. Optimal killing activity was observed with tyrosinase in P2, MAGE-2 in P3 (99.7 ± 5.9%, $P < 0.01$ compared with control; 56.2 ± 2.5%), and MAGE-3 in P5 (80.6 ± 3.4%, $P < 0.01$ compared with control; 54.6 ± 0.9%) at an effector-to-target ratio of 10. P1 and P3 also showed killing activity against HLA-A*0201-restricted target cells pulsed with Melan-A peptide (data not shown). The cytolytic activity was always detected in cultures which successfully proliferated on stimulation with immunizing peptide. CTLs from P4 could not be expanded to the level necessary for killing assays. Therefore, the therapeutic efficacy of PPI seemed to be correlated with *in vitro* effective propagation of peptide-specific CTLs with apparent cytolytic function.

Clinical efficacy of PPI for melanoma treatment. A critical issue is the therapeutic potency of PPI-induced CTLs. Therefore, the tumor response associated with the first round of therapeutic PPI was evaluated in P3 to P8 who had measurable lesions (Table 1). In P3, an enlarged mediastinal lymph node due to metastasis of melanoma cells decreased in size, from the longest diameter of 3.2 cm before PPI, to 0.9 cm at completion of PPI (Fig. 4*A*). P5 showed a >50% reduction in the sums of the longest

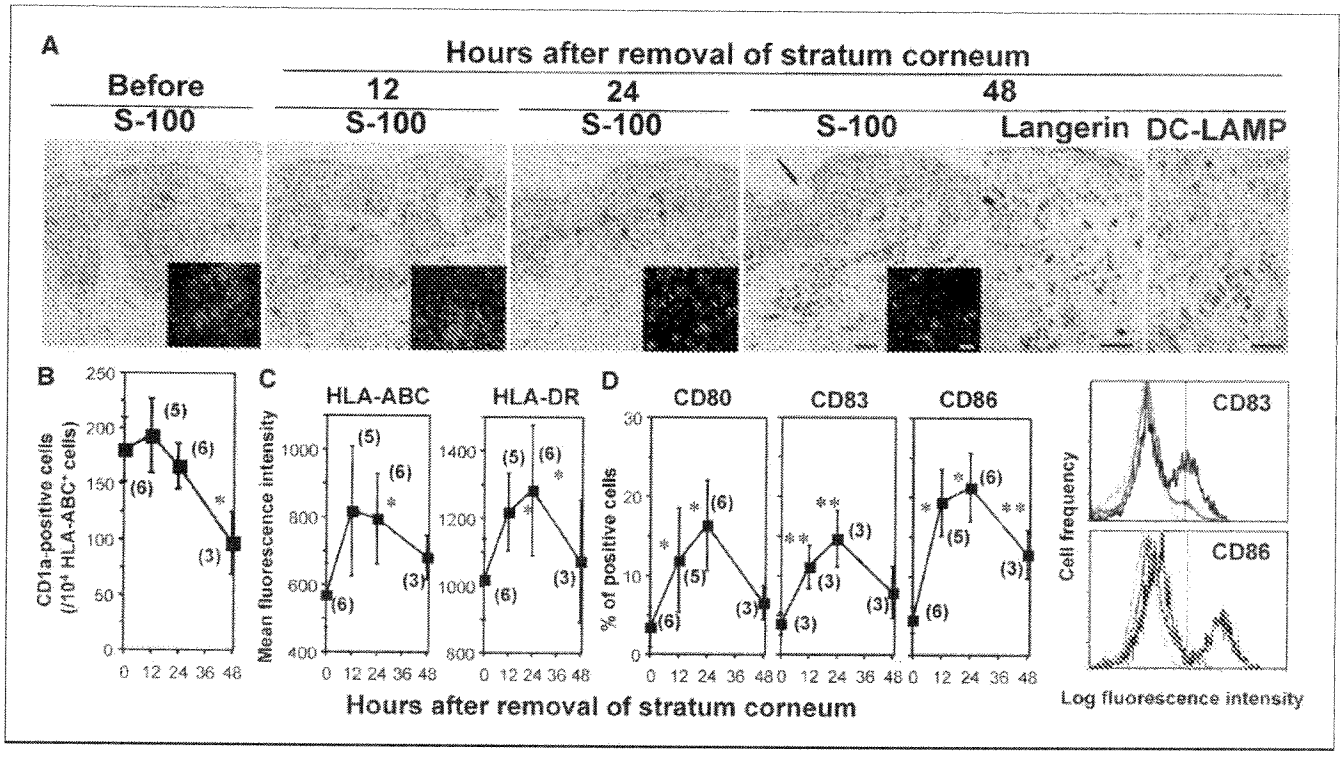

Figure 1. Kinetics of LCs and expression of HLA and costimulatory molecules on LCs in relation to removal of the SC. Skin samples from normal individuals were examined before, 12, 24, and 48 hours after removal of the SC. The numbers in parentheses indicate the number of subjects that participated. *A*, $S\text{-}100^+$, Langerin/$CD207^+$ and DC-LAMP/$CD208^+$ cells (*brown*) in the skin samples immunostained with a standard avidin-biotin complex method. Nuclear counterstaining was achieved with hematoxylin. *Arrow*, regenerating SC. *Insets*, composite confocal images of epidermal sheets showing HLA-DR^+ cells. Representative sections are shown. *Bar*, 50 μm. *B*, enumeration of $CD1a^+$ cells in HLA-ABC^+ epidermal cells. *Points*, mean; *bars*, ±SD. *C* and *D*, expression profiles of HLA-ABC, HLA-DR, CD80, CD83, and CD86 in epidermal LCs. *C*, mean fluorescence intensity was determined as a linear scale (0-1,020). *D*, percentage of positive cells in LC populations were determined. LCs were separated from contaminating epidermal cells using FL2-gate settings as PE-labeled $CD1a^+$ cells and FL3-gate settings as PerCP-labeled HLA-DR^+ cells, as well as forward scatter/side scatter gating. The viability of the cells within this gate was always >95% as determined by the addition of propidium iodide to each sample. Representative flow cytometric analyses of cell surface CD83 and CD86 expression before (*red lines*) and 24 hours after (*black lines*) the barrier disruption. Cells stained with isotype control antibody (*green lines*). *Points*, mean; *bars*, ±SD; *, $P < 0.01$; **, $P < 0.05$, as compared with no treatment (0 hours).

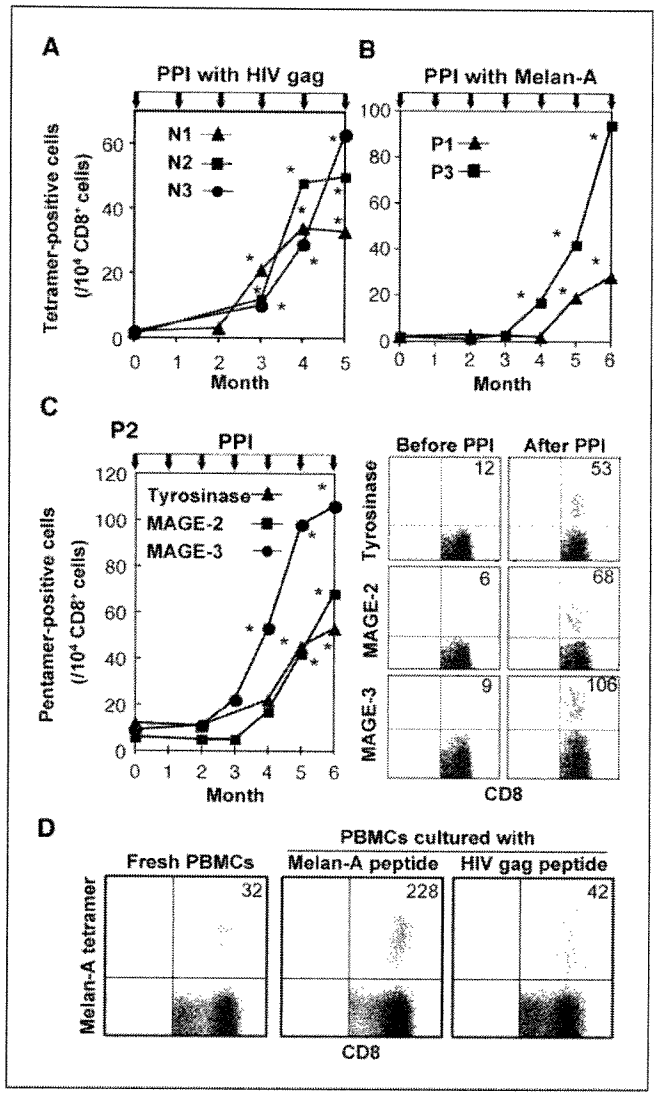

Figure 2. Induction of peptide-specific, tetramer-positive, and pentamer-positive CD8⁺ T cells. PBMCs obtained 7 days after each PPI were immunophenotyped with the HIV gag-tetramer in N1 to N3 (*A*), Melan-A-tetramer in P1 and P3 (*B*), and pentamers for tyrosinase, MAGE-2, and MAGE-3 in P2 (*C*), and analyzed by flow cytometry. Alternatively, cells were cultured with Melan-A or HIV gag peptide for 5 days and subjected to tetramer binding assay (*D*). *A*, kinetics of HIV gag tetramer-positive cells. The value for HIV gag-specific cells/10^4 CD8⁺ cells in HLA-A*0201 control subjects ($n = 6$) without PPI was 1.83 ± 0.75 (mean ± SD). *,+2 SD above the mean. *B* and *C*, kinetics of tetramer/pentamer-positive cells in melanoma patients. The number of Melan-A-specific cells/10^4 CD8⁺ cells in HLA-A*0201 control subjects ($n = 6$) without PPI was 2.50 ± 1.05 (mean ± SD). The number of tyrosinase-, MAGE-2-, and MAGE-3-specific cells/10^4 CD8⁺ cells in HLA-A*24 control subjects ($n = 6$) without PPI were 10.2 ± 5.9, 11.3 ± 5.0, and 15.6 ± 3.8, respectively (mean ± SD). *,+2 SD above the mean. *D*, specificity of tetramer-positive cell induction by PPI. Results of a representative experiment for cells after the seventh immunization with Melan-A from P1. Numbers are tetramer-positive cells/10^4 CD8⁺ cells. Tetramer-positive cells in cultures stimulated with nonimmunizing peptide were always below the mean +2 SD of control subjects ($n = 6$).

diameters of five target lesions selected before PPI (Fig. 4*B*). In P7, a small s.c. nodule, with the longest diameter at 2 cm, disappeared. In P8, the intensities of the scintigraphic signals in five of six multiple bone metastases were attenuated after PPI (Fig. 4*C*). In accordance with these findings, the level of serum 5-S-cysteinyl-dopa was reduced from 27 nmol/L before PPI to 4.2 nmol/L after PPI (normal, <8.0 nmol/L). In P4 and P6, the melanoma invasion progressed rapidly, despite CTL induction, and both patients died of the tumor within 4 months of PPI completion. In P2 and P7, although skin nodules had developed at frequencies of one and three per 2 months, respectively, and been dissected during the 6 months prior to PPI, new lesions did not appear for 6 months during PPI. These patients developed several new lesions at

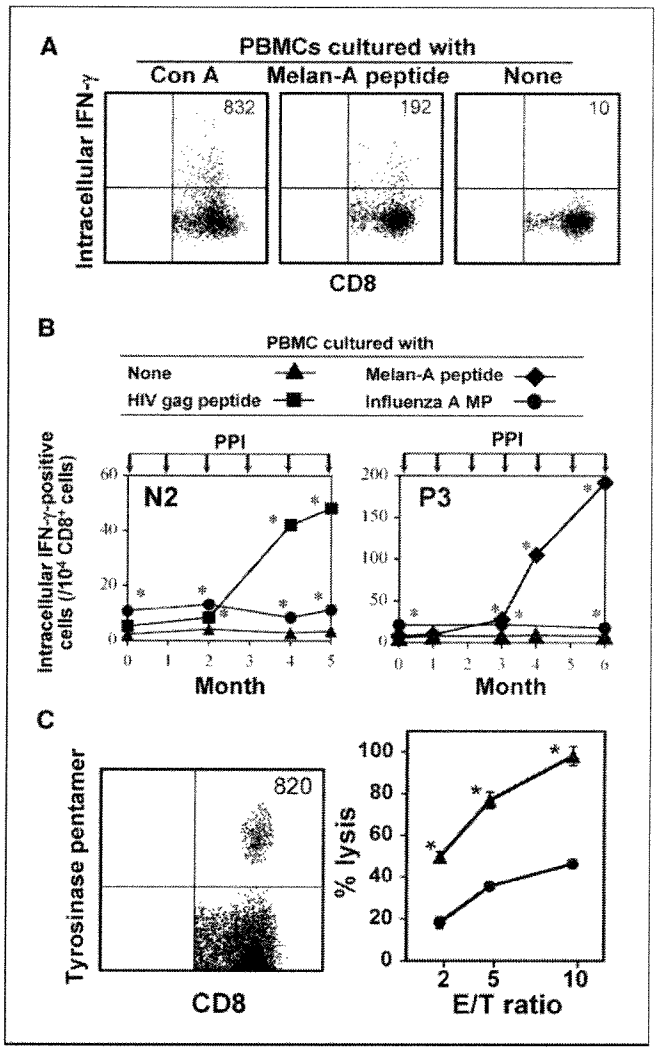

Figure 3. Induction of an intracellular IFN-γ⁺ CD8⁺ T cell response by PPI. PBMCs obtained 7 days after PPI were cultured with either HIV gag, Melan-A, influenza A MP, or Con A for 48 hours depending on the experiment purpose and subjected to intracellular IFN-γ assay. *A*, specific induction of intracellular IFN-γ-positive cells by PPI. Results of a representative experiment in P3 after the seventh immunization. Numbers indicate IFN-γ⁺ cells/10^4 CD8⁺ T cells. In the Con A culture, these were 320 in N1, 343 in N2, 425 in N3, and 892 in P1. *B*, kinetics of peptide-specific T cells producing IFN-γ. Results of representative experiments in N2 and P3. Immunizing peptide was HIV gag in N2 and Melan-A in P3. The number of HIV gag–specific cells/10^4 CD8⁺ cells in HLA-A*0201 control subjects ($n = 6$) without PPI was 3.86 ± 1.77 (mean ± SD). The number of Melan-A-specific cells/10^4 CD8⁺ cells in HLA-A*0201 control subjects ($n = 5$) without PPI was 4.17 ± 1.60 (mean ± SD). *,+2 SD above the mean. *C*, results of representative *in vitro* peptide-dependent cytolytic experiments in P2. PBMCs at completion of PPI were stimulated with tyrosinase and subjected to CTL assay in the presence of T2-A24 target cells loaded with (▲) or without (●) the peptide. Effector cell populations contained 820 tyrosinase pentamer-positive cells/10^4 CD8⁺ cells as shown in a flow cytometric profile. *Points*, mean of triplicate cultures; *bars*, ±SD. *, $P < 0.01$ as compared with lysis of T2-A24 cells not loaded with the relevant peptide. *E/T ratio*, effector-to-target ratio.

3 to 5 months after completion of PPI, once the number of circulating CTLs had dropped to less than half the peak value (data not shown). The fact that P2 and P7 were free from melanoma lesions for 9 to 11 months during and after PPI suggested the suppressive effect of this procedure on tumor development. Therefore, the clinical outcomes in this small pilot study clearly suggest the beneficial effects of PPI for patients with advanced melanoma.

Migration of CTLs into melanoma lesions after PPI. Skin tissue specimens were available in association with PPI for P2 and P4, and subjected to histologic and immunohistochemical examinations as well as flow cytometric analysis. In P4, in whom the melanoma progressed despite CTL induction in the blood, there was no lymphoid cell infiltration before or after PPI (Fig. 5*A*, *a*). In P2, the metastatic lesions contained no cellular infiltrate before the first round of PPI (Fig. 5*A*, *b*). This patient received a second round of PPI due to the development of three new lesions at 3 months after completion of the first round of PPI. After the second immunization in the second round, the regressing lesions showed marked lymphoid cell infiltration and apoptotic and necrotic melanoma cells (Fig. 5*A*, *c* and *d*). $CD8^+$ cells had infiltrated the tumor mass and $CD4^+$ cells were located at the periphery of the lesions (Fig. 5*A*, *e* and *f*). Flow cytometric analysis revealed that the tumor-infiltrating leukocytes contained $CD4^+$ cells and $CD8^+$ cells in equal proportions (Fig. 5*B*). Furthermore, 9.8%, 8.4%, and 12.7% of the cells among the $CD8^+$ tumor-infiltrating cells were positive for tyrosinase, MAGE-2, and MAGE-3 pentamers, respectively (Fig. 5*B*). The proportions of tyrosinase, MAGE-2, and MAGE-3 pentamer–positive cells in the circulation at this time were 0.42%, 0.52%, and 0.8% of the $CD8^+$ cells, respectively, indicating selective migration of peptide-specific CTLs into the melanoma lesions.

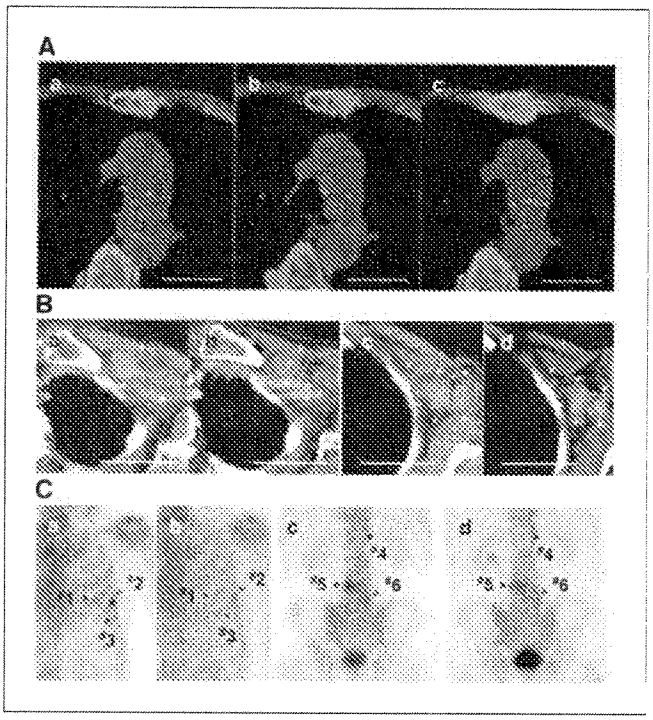

Figure 4. Clinical efficacy of PPI for melanoma treatment. *A*, computed tomography scans of a mediastinal lymph node (*arrowheads*) in P3 on entry (*a*), after the fourth PPI (*b*), and at completion of PPI (*c*). *B*, multiple metastatic lesions (*arrowheads*) in the left upper chest (*a* and *b*) and left axilla (*c* and *d*) in P5 on entry (*a* and *c*) and at completion of PPI (*b* and *d*). *C*, ^{99m}Tc-bone scintigraphs of metastatic bone lesions (#1-#6) in P8 before (*a* and *c*) and after (*b* and *d*) PPI. P3 presented with a melanoma lesion on the left palpebral conjunctiva and received orbital exenteration and wide local excision 15 years previously. Metastatic lesions appeared on the skin of the neck and the left parotid gland >12 years after the previous treatment. Despite total excision of the recurrent lesions and postoperative combination adjuvant therapy, metastases developed in mediastinal lymph nodes 3 months before entry. P5 underwent excision of primary skin lesions 5 years previously without any subsequent treatment, and developed multiple s.c. metastatic tumors at 5 months before entry. P8 had multiple bone metastases, as detected by bone scintigraphy, despite the resection of a lesion of the first toe of the right foot and right inguinal metastatic lymph nodes, and subsequent chemotherapy 2 years before entry.

Laboratory findings and untoward clinical features. Repeated assessment of hemograms and liver and renal functions revealed no abnormalities during and at completion of PPI in normal participants and the patients except P4. Results of the laboratory examination were normal during the 1-year follow-up period after completion of PPI in N1 to N3. Liver functions progressively deteriorated during PPI and until death in P4 because of multiple liver metastases. N1 to N3 developed no physical signs of autoimmunity, and rheumatoid factors or autoantibodies including antinuclear antibodies were negative 1 year after PPI. Generalized progressive vitiligo spots developed in P1, P3, P5, and P6 after several immunizations, although no other physical signs or serologic findings of autoimmunity were detected at completion of PPI.

Follow-up. P1 and P3 were disease-free for 15 to 19 months after the first round of PPI. New small s.c. nodules in P2 and P7, which developed at 3 and 5 months, respectively, after the first round of PPI, disappeared after a second round of PPI. Despite the regression of the initial target lesions at the end of PPI, P5 developed new s.c. and metastatic lesions while undergoing monthly immunization in succession to the PPI due to the patient's request. P8 developed a new bone lesion with an increase in the serum 5-S-cysteinyldopa level at 4 months after PPI, although the densities of the previous bone lesions remained decreased, as evaluated by scintigraphy.

Discussion

The present study showed that PPI, a novel immunization protocol for peptide application to barrier-disrupted skin, induces potent CTL responses and provides a promising approach for cancer therapy in human beings. Because an adjuvant effect seems to be inherent in perturbation of the skin integrity (21), the hallmark of PPI is that it closely mimics the natural trigger of DC activation, conducive to CTL expansion as fully activated effector cells. Although only a part (a maximum of 20%) of LCs were actually activated, the main and effective inducer of CTL would be LCs, considering their *in situ* kinetics after removal of SC in human and the augmented CTL priming capacity of LCs in barrier-disrupted murine skin (20). Recent studies in murine herpes virus infection models (34, 35), as well as novel mouse systems in which LCs can be selectively removed/ablated (36–38), have suggested that skin-resident APCs other than LCs contribute in the generation of immune responses *in vivo*. Therefore, it was possible that these cell types also participated in peptide-specific CTL immunity in the current protocol. Individual differences in the LC emigration profile of these APCs through skin and the amount of peptide absorbed via barrier-disrupted skin may be one critical factor in determining the timing and magnitude of CTL responses. An important issue is the demonstration of an actual therapeutic or preventive application of PPI in humans. In fact, the pilot study revealed a beneficial effect of PPI on the growth inhibition of

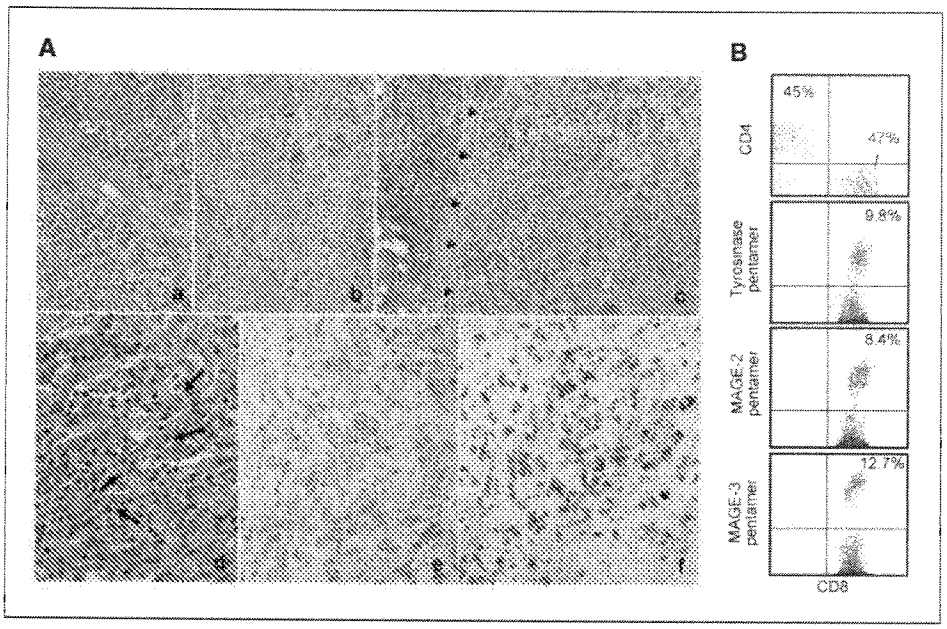

Figure 5. *In vivo* infiltration of CTLs into melanoma lesions induced by PPI. *A*, H&E histology and immunohistology of metastatic skin lesions. Skin lesions from P4 after PPI (*a*, magnification, ×200), from P2 before (*b*, magnification, ×200) and after PPI (*c*, *arrowheads*, tumor mass; magnification, ×100), with apoptotic and necrotic cells (*d*, *arrows*; magnification, ×400) and immunohistochemical staining showing infiltration of $CD4^+$ cells (*e*, magnification, ×100) and $CD8^+$ cells (*f*, magnification, ×100). *B*, flow cytometric profiles of cell suspensions obtained from a regressing s.c. nodule in P2 during PPI, showing the percentage of $CD4^+$ and $CD8^+$ cells among $CD45^+$ cell-gated leukocytes and the percentage of $pentamer^+$ cells among the $CD8^+$ $CD45^+$ cell-gated populations.

tumors in patients with advanced melanoma, which accompanied the induction of specific CTL activity. None of the patients/controls who received PPI exhibited local or systemic toxicity, or developed any clinical and laboratory findings of autoimmunity except vitiligo in patients with melanoma. We therefore propose that this novel strategy is clinically relevant for application in the treatment of malignancies. Furthermore, the generation of HIV gag–specific CTLs indicates the prophylactic strategy of PPI against viral and helminth infections.

Methods of needle-free vaccination delivery have attracted a global interest because of the urgent need for eradication of pandemic disease and treatment of growing numbers of cancer patients. This new approach has several advantages regarding ease and speed of delivery, safety and compliance, and costs, over needle delivery. Reported needle-free strategies to manipulate primarily the skin immune system include transcutaneous immunization (TCI; refs. 19, 39, 40), penetration via hair follicles (41), cutaneous bombardment (42), epidermal powder immunization (43, 44), and immunization with microenhancer arrays (45). All of these protocols target antigen to skin DCs in association with their activation and emigration from the skin, regulating the magnitudes, types, and directions of the immune responses. In particular, TCI is close to PPI in the methodologic respect. Both TCI and PPI are characterized by the application of antigen to the skin surface, thereby treating pathologic processes at a location distant from the application site. The difference between these two methods is the use of adjuvant. To obtain satisfactory immune responses, TCI requires adjuvant such as cholera toxin added to a vaccine antigen. On the other hand, barrier disruption is mandatory to allow the antigen to penetrate and activate the skin immune system in PPI without adjuvant. Although comparison of the effectiveness of CTL induction between PPI and other transcutaneous methods is difficult at present, the current study is the first one that clearly shows the clinical efficacy of PPI-induced CTLs in the human system.

Among many variables in the protocols and technologies of DC immunotherapy, quality control of vaccines in relation to the maturation status of DCs is a key determinant for the regulation of immune responses, and thus, clinical efficacy (4). Barrier disruption with the strong glue in PPI constantly removed a definite amount of the horny layers, irrespective of age, gender, and treated sites of the recipient. Such reproducibility of barrier perturbation enabled us to use *in situ* activated and fully matured LCs as therapeutic vectors. Repeated manipulations were essential to induce CTL responses with clinical efficacy in PPI as in prevailing melanoma vaccine with DC preparations (6). Such a time-consuming strategy might pose a problem when rapid protective responses are required. The application of appropriate adjuvants to the sites of barrier disruption has been shown to enhance immune responses both in mice (19, 39) and in humans (40). Systemic and local incorporation of T cell adjuvants such as interleukin-2, IFNs, and CD4 epitopes to PPI may potentiate CTL induction with less frequent immunization.

In the present pilot study, PPI was in fact effective in patients with advanced melanoma because tumor size was reduced in four of six patients, and apparent tumor burden and tumor development seemed to be abrogated in another. These beneficial effects coincided with the emergence of CTLs with strong cytolytic activity in the blood of some patients. Regressing lesions following PPI was associated with preferential infiltration of CTLs in a responder patient. In contrast, one patient with multiple metastases, and another with primary esophageal melanoma, did not clinically respond to PPI despite the presence of circulating CTLs. The possible reasons for treatment failure seemed to be related to the lack of cellular infiltrate in lesions and the impaired ability of CTLs to propagate *in vitro* under antigenic stimulation. A variety of immunologic mechanisms to evade tumor cell killing might underlie such T cell defects in these patients (46–48).

The safety issue is an important concern because the development of autoimmunity has been reported with the introduction of antigens directly into the body (49). Vitiligo, a well-known feature of autoimmunity targeting melanocytes, develops in association with DC-based immunotherapy in melanoma cases (49, 50). Because the antigens used for the immunization are autoantigens, there would be no expectation of epitope spreading. Although no study participants undergoing PPI

showed any signs of autoimmunity other than vitiligo, careful and repeated follow-up of the recipient's physical condition is indispensable for clinical trials.

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